sarcomere length (SL). In this study, we used time-resolved in situ FRET to monitor the effects of Ca^{2+}-occupancy, XB state, and SL on N-cTnC opening in skinned cardiac muscle fibers. FRET donor (AEDANS) and acceptor (DDPM) modified double-cysteine mutant cTnC(13C/51C)AEDANS-DDPM was reconstituted into skinned muscle fibers to examine the N domain of cTnC (N-cTnC) opening. To study the effect of SL on structural transitions of cTnC, we monitored the protein structural transitions at low and high [Ca^{2+}] and SL 1.8 and 2.2 μm. Mg^{2+}-ADP and sodium orthovanadate (Vi) were used to examine the effects of non-cyclng strong and weak XBs, respectively. We found that strongly bound XBs alter structural transitions of cardiac troponin only at 2.2 μm. On the other hand, Vi blunted the SL dependent opening of N-cTnC such that weak XBs have no effect on N-cTnC at either [Ca^{2+}] or SL. In addition, distance distribution analysis indicated that N-cTnC adopts four unique conformations associated with the four states of thin filament regulation, and that N-cTnC conformational equilibria are caused by cycling XBs. Based on our findings, we conclude that the observed dependence of myosin positive feedback regulation on SL is an important determinant of the Frank-Starling law of the heart.

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Ca^{2+}-Regulatory Function of the Inhibitory Peptide Region of Cardiac Troponin I is Aided by the C-Terminus of Cardiac Troponin T: Effects of FHC Mutations Ctni R145G and Ctnt R278C, Alone and in Combination, on Filament Sliding

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Investigations of cardiomyopathy mutations in Ca^{2+} regulatory proteins troponin and tropomyosin provide crucial information about cardiac disease mechanisms, and provide insights into functional domains in the affected polypeptides. Hypertrophic cardiomyopathy-associated mutations TnI R145G, located within the inhibitory peptide (I_p) of human cardiac troponin I (hcTnI), and TnT R278C, located immediately C-terminal to the IT arm in human cardiac troponin T (hcTnT), share remarkable features: structurally, biochemically, and pathologically. Using bioinformatics, we find compelling evidence that affected regions of hcTnI and hcTnT, may be related_not just structurally_but also evolutionarily. Because alignment of TnI and TnT coincides with the known structure of the IT-arm, and both Arg mutations are located close to the C-terminal end of the IT-arm, we investigated functional relationships between hcTnI R145G and hcTnT R278C. We hypothesized that if the mutations affected function independently, then their effects would be additive in a double mutant complex. We characterized Tn complexes containing either mutation alone, or both mutations simultaneously, using in vitro motility assays run with varying [Ca^{2+}], temperature, or HMM density. Our most significant findings show that TnT R278C "rescued" some deleterious effects of TnI R145G at high Ca^{2+}, but exacerbated the loss of function (i.e., switching off the actomyosin interaction) at low Ca^{2+}. Taken together, our results raise the likelihood that cTnI's I_p sequence might share a common evolutionary origin with, and thus be structurally and functionally related to, the C-terminus of cTnT. In accord with this prediction, our experimental results suggest that the C-terminus of cTnT aids Ca^{2+}-regulatory function of cTnI I_p within the troponin complex.

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Impact of Troponin-I Phosphorylation on Human Cardiac Myofilament Function

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A long-term goal of work in our laboratories is to understand the structural basis of myofilament function, i.e. cross-bridge dynamics (CD) and myofilament length dependent activation (LDA) in striated muscle in heath and disease. Moreover, in cardiac muscle LDA underlies the Frank Starling Law of the Heart. Troponin phosphorylation, and in particular troponin-I (cTnI), has been suggested to be a pivotal modulator of myofilament function. Here we examined the impact of phosphorylation of distinct cTnI domains on CD and LDA in isolated human myocardium. Site specific phosphorylation was accomplished by charge mutation of hcTnI phosphomimics on the PKA (S23/24D), PKC (S42/44E; T143E), AMPK (S150D), and novel (S5/6D) sites, followed by recombinant protein exchange into skinned non-failing human LV cardiac muscle strips (~2mm long, and ~150um diameter. Force and ATPase activity was measured as function of [Ca^{2+}] at short and long sarcomere length (SL=2.0&2.3um). We found, LDA: hcTnI-S150D attenuated, hcTnI-S42/43E increase, and no effect for the other sites. CD as indexed by tension cost was: decreased for cTnI-S42/44E and hcTnI-S150D, and no effect for the other sites. We conclude that cTnI phosphorylation at distinct sites differentially affect cross-bridge cycling and length dependent activation in human myocardium. Structural analysis employing x-ray diffraction is underway to determine the structural basis for these phenomena. Supported by NIH HL075494, HL62426, GM103622.

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The R144W Mutation in Mouse Cardiac Troponin T Attenuates Cross-bridge Recruitment and Detachment Kinetics

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A missense mutation, R141W, in the strong tropomyosin-binding region of human cardiac troponin T (cTnT) is associated with dilated cardiomyopathy (DCM). Previous studies of steady-state contractile function suggest that DCM-related mutations in cTnT attenuate myofilament Ca^{2+} sensitivity. Steady-state observations by themselves may not be sufficient enough to provide a reliable link between different mutations and divergent cardiac phenotypes, especially at submaximal Ca^{2+} levels. It is now widely appreciated that dynamic relationships - rather than steady-state aspects of the force-pCa relationship - dominate in conditions under which cardiac muscle functions. To understand the effects of the R141W mutation on cardiac contractile dynamics, we created a mouse cTnT analog ($McTnT_{R144W}$) of the human mutation, R141W. $McTnT_{R144W}$ and the wild-type McTnT were individually reconstituted into detergent-skinned mouse cardiac muscle fibers and dynamic contractile features were assessed at maximal (pCa 4.3) and submaximal (pCa 5.5) activations. $McTnT_{R144W}$-reconstituted fibers revealed the following. The speed of crossbridge (XB) recruitment, *b*, decreased significantly at both pCa 4.3 and pCa 5.5; however, the magnitude of decrease was 2-fold greater at submaximal activation. The speed of XB detachment dynamics, *c*, also decreased and was 1.7-fold greater at submaximal activation. However, the XB strain-mediated effects on the recruitment of other XBs (γ) - mediated by allosteric/cooperative mechanisms operating within the thin filament - decreased to a similar extent at both Ca^{2+} activations. Novel findings from our study will be discussed in terms of the $McTnT_{R144W}$-induced effects on the thin filament cooperativity and its associations with slower rates of XB recruitment and detachment kinetics at physiologically relevant Ca^{2+} concentrations.

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Effects of Pseudophosphorylation of Rat Cardiac Troponin T Residue 204 are Differently Affected by α- and β-Myosin Heavy Chain Isoforms

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We tested our hypothesis that α-myosin heavy chain (MHC) and β-MHC differently modulate the functional effects of protein kinase C (PKC)-mediated phosphorylation of rat cardiac troponin T (RcTnT). We generated a chimeric pseudophosphorylated RcTnT in which the threonine 204 was replaced by glutamic acid ($cTnT_{T204E}$) to mimic the PKC-mediated phosphorylation effect. Recombinant proteins were reconstituted into detergent-skinned cardiac muscle fibers from normal rats expressing α-MHC or propylthiouracil-treated rats expressing β-MHC. Steady state measurements revealed that Ca^{2+}-activated maximal tension and the corresponding ATPase activity decreased significantly by ~75% in α-MHC+$cTnT_{T204E}$ fibers, but only by ~33% in β-MHC+$cTnT_{T204E}$ fibers. However, the myofilament Ca^{2+} sensitivity (pCa_{50}) decreased by ~50% in both α-MHC+$cTnT_{T204E}$ and β-MHC+$cTnT_{T204E}$ fibers, suggesting that the greater decrease in maximal tension observed in α-MHC+$cTnT_{T204E}$ fibers cannot be merely attributed to the decrease in Ca^{2+}-mediated activation of thin filaments. Interestingly, the rates of tension redevelopment (k_{tr}), crossbridge (XB) recruitment dynamics (*b*), XB distortion dynamics (*c*), and the tension cost (ATPase rate/tension) decreased only in α-MHC+$cTnT_{T204E}$ fibers. Our results demonstrate that α- and β-MHC isoforms have different impact on